REMARKS

The Official Action dated April 8, 2003 and the references cited therein have been fully considered. Reconsideration of this application as amended is respectfully requested in light of the foregoing amendments and following remarks.

Claims 1-41 are pending in the application.

Claims 6, 8, 10 and 14 are amended herein.

Claims 16-41 are canceled without prejudice as they have been withdrawn from further consideration as being drawn to non-elected embodiments of the invention. Applicants reserve the right to file a divisional application to the non-elected embodiments.

Claims 1-15 are presented for consideration.

Sequence Requirements

The Office Action contains an objection to the Sequence Listing provided by Applicants. However, with the exception of a missing sequence identifier for claim 8, no specifics were provided as to the nature of the failure to comply with the requirements set forth in 37 CFR §§1.821-1.825. A telephone call placed to the sequence Rules Interpretation unit (identified in the Notice To Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures) confirmed that the computer readable and paper copy of Applicants' Sequence Listing were acceptable. Applicants submit that while the language of claim 8 failed to include the sequence identifier, the sequence of claim 8 was included in the Sequence Listing and the sequence identifier was included within the specification, i.e. SEQ ID NO: 1, at page 11, line 26. Claim 8 has been amended to include the sequence identifier and, as such, no additional sequence needs to be added to the Sequence Listing which would necessitate a substitute computer readable and paper copy thereof. If there are additional deficiencies, Applicants will submit a corrected version upon the identification of same.

Information Disclosure Statement

The Office Action states that the file wrapper for this application indicates that an IDS was submitted, but it cannot be found. According to Applicants' copy of this file, an IDS

has not been previously submitted. Applicants have submitted an IDS and the form PTO-1449 with this response.

Claim Objections

Claims 10-13 are objected to as being in improper dependent form. Applicants have amended claim 10 to address this objection. It should be noted that the instant invention features helper-dependent adenoviral vector elements and helper adenoviral elements that enhance the product and isolation of helper-dependent adenoviral vectors. As such, the claimed nucleic acid of claim 10 is one of said elements. The amendment has been made to further distinguish the claimed invention. Applicants request that this objection be withdrawn in view of the amendment made herein.

Claim Rejections Under 35 U.S.C. §112

Claims 1-15 are rejected under 35 USC §112, second paragraph as being indefinite with respect to the phrase "low homology." Applicants have provided within the Specification on page 4, lines 1-7, a definition for the phrase "low homology." Likewise, Claim 10 is rejected under this section as being indefinite with respect to the phrase "helper virus." Applicants have provided within the Specification, beginning on page 2, line32, through page 3, line 4, a definition for what is meant by "helper virus." This definition, along with the amendment to claim 10, adequately defines the metes and bounds of the claim. Similarly, with respect to the assertion that claim 7 is indefinite in view of the phrase "2-3 times less efficient", Applicants have defined what is meant by "less efficiency" on page 4, lines 8-15 of the Specification.

With respect to claim 8, it has been asserted that Applicants have given a meaning to a claim element that is contrary to its accepted use in the art, i.e. "A element" being defined as $ATTTGN_8GC$, relying on Grable et al.'s 1992 paper in the J. of Virology. However, Applicants have used the term "A element" consistently with the understanding of those skilled in the art. A later publication of Patrick Hearing, who was also a principal author on the Grable et al. paper, redefines $ATTTGN_8GC$ as the consensus sequence, which was more complex than the earlier published motif. See, Schmid, S. and Hearing, P., J. of Virology, Vol. 71, No. 5, p. 3375-3384 (1997) (a copy of which is enclosed with this response) and more specifically, Fig. 4 and page

3383, Col. 1, third paragraph. The Schmid and Hearing paper was referenced in the Specification, page 11, line 16, with a definition provided of what Applicants meant by the term "A element."

In view of the above, it is respectfully requested that this rejection be withdrawn.

Claim Rejection Under 35 U.S.C. §102

Claims 1-5 and 7 are rejected under 35 U.S.C. §102(b) in view of Hardy et al. (WO 97/32481). It has been asserted that said reference teaches the low homology packaging signal of the instant invention. Applicants respectfully traverse this rejection in that any example of packaging signals having less than 100 percent homology taught by Hardy are not identical to the claimed invention. As noted above, Applicants have clearly described what they intended to be encompassed by the term "low homology" with the definition provided on page 4, lines 1-8 of the Specification. "Low homology" has been defined as a stretch of DNA within the packaging region that is no more than 25 bp, and preferably 23 bp, of contiguous sequence homology between the native Ad5 and the modified helper virus. This was accomplished by altering the eight ambiguous nucleotides within each of the seven "A elements", i.e. repeat units, according to the consensus sequence 5'-TTTGN$_8$GC-3 (SEQ ID NO:1). The sequence of eight ambiguous nucleotides was designed by taking these eight nucleotides from several A elements and replacing them with a sequence taken from a different A element such that, for example, the A1 unit has its N$_8$ region replaced with the N$_8$ region from the A5 unit and the N$_8$ region from the A2 unit was replaced by those from the A6 unit. In addition, new elements were created by modifying the existing nucleotides to the consensus sequence or by exchanging nucleotides therein. There is no teaching, either expressed or implicit, of modifications to the packaging signal in Hardy that teach or suggest this modification such that Hardy would anticipate the claimed invention.

Claims 6, 8 and 9-12 are rejected under 35 U.S.C. §102(b) in view of Hardy. It has been asserted that Hardy teaches the use of heterologous packaging signals from Ad7 in place of wild-type Ad5 packaging signals that are equivalent to the packaging signals of the claimed invention. It is further asserted that this reference teaches plasmids which can contain E1 deletions, be a helper virus and accommodate insertions of about 2.9Kb. Applicants respectfully traverse this rejection.

It should be noted that the only independent claim of the instant application under consideration (claim 1) is directed to a novel element that has been incorporated into known helper virus vectors. Specifically, as discussed above, the novel element is a modified packaging signal having low homology to, and less activity than, a wild-type packaging signal. The modified packaging signal is used in a helper virus to decrease recombination and generation of the virus. The remainder of the pending claims and, more specifically, claims 6, 8, and 9-12 are dependent claims directed to additional features of the invention, which by the nature of their dependency would include the novel element in claim 1. Applicants do not refute the assertions made in the Office Action that Hardy discloses the use of Ad7 packaging signals, constructs which can be plasmids, which can contain deletion of the E1 region or be a helper virus and/or which may accommodate inserts of about 2.9 Kb. However, Hardy does not teach any construct having the modified packaging signal of claim 1 either alone or in combination with the additional features of dependent claims 6, 8 and 9-12 such that it would anticipate the claimed invention.

Claim 14 is rejected under 35 U.S.C. §102(b) in view of Graham (U.S. Pat. No. 5,919,676). It has been asserted that Graham teaches an E3 containing nucleic acid that can have an insertion of 2.7 Kb. Applicants respectfully traverse this rejection. Claim 14 as amended is a dependent claim and, as such, would incorporates the features of the claim(s) from which it depends. As such, the claim is directed to a nucleic acid molecule having the modified packaging signal (of claim 1) and the E3 gene having an insert of about 2.7 Kb, but does not contain a promoter sequence. Graham does not teach any construct having the modified packaging signal of claim 1 either alone or in combination with the E3 gene of claim 14 such that it would anticipate the claimed invention.

Thus, it is respectfully submitted that the cited references do not anticipate the claimed invention. Applicants request that the Examiner reconsider and withdraw this rejection.

Claim Rejection Under 35 U.S.C. §103

Claims 6, 8, 9 and 12-13 are rejected under 35 U.S.C. §103(a) as being unpatentable over Hardy. It has been asserted that Hardy teaches a nucleic acid having packaging signals with less than 100% homology, i.e. low homology and E3 regions having insertions. Applicants respectfully traverse this rejection. As noted above, there is no disclosure

in Hardy as to the modified packaging signal feature claimed by the Applicants and incorporated by the dependency of claims 6, 8, 9 and 12-13. As such, contrary to the assertions in the Office Action, there is no disclosure in Hardy that would motivate or suggest to even one highly skilled in the art of genetic manipulation to make the nucleic acid molecule with the modified packaging signal and the features of these claims.

Claim 15 is rejected under 35 U.S.C. §103(e) as being unpatentable over Graham. It has been asserted that Graham teaches the insertion of DNA, but admittedly does not teach the insertion of introns. Applicants respectfully traverse this rejection. As with the discussion of Hardy above, there is no disclosure in Graham as to the modified packaging signal feature claimed by the Applicants and incorporated by the dependency of this claim. As such, contrary to the assertions in the Office Action, there is no disclosure in Hardy that would motivate or suggest to one of ordinary skill in the art to make the nucleic acid molecule with the modified packaging signal the feature of this claim.

Thus, it is respectfully submitted that the cited references do not render the instant invention obvious and Applicants request that the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. §103.

CONDITIONAL PETITION

Applicants hereby make a Conditional Petition for any relief available to correct any defect in connection with this filing, or any defect remaining in this application after this filing. The Commissioner is authorized to charge deposit account 13-2755 for the petition fee and any other fee(s) required to effect this Conditional Petition.

CONCLUSION

In view of the foregoing amendments and remarks, it is seen that all grounds of rejection have been overcome and that Claims 1-15 are in proper condition for allowance. Accordingly, Applicants respectfully request that all of the objections and rejections of record be withdrawn and that a Notice of Allowance be forwarded to the Applicants. An early Office Action to that effect is, therefore, earnestly solicited. The Examiner is invited to contact Applicants' Attorney at the telephone number given below, if such would expedite the allowance of this application.

Respectfully submitted,

By _____

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Bipartite Structure and Functional Independence of Adenovirus Type 5 Packaging Elements

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Selectivity and polarity of adenovirus type 5 DNA packaging are believed to be directed by an interaction of putative packaging factors with the *cis*-acting adenovirus packaging domain located within the genomic left end (nucleotides 194 to 380). In previous studies, this packaging domain was mutationally dissected into at least seven functional elements called A repeats. These elements, albeit redundant in function, exhibit differences in the ability to support viral packaging, with elements I, II, V, and VI as the most critical repeats. Viral packaging was shown to be sensitive to spatial changes between individual A repeats. To study the importance of spatial constraints in more detail, we performed site-directed mutagenesis of the 21-bp linker regions separating A repeats I and II, as well as A repeats V and VI. The results of our mutational analysis reveal previously unrecognized sequences that are critical for DNA encapsidation in vivo. On the basis of these results, we present a more complex consensus motif for the adenovirus packaging elements which is bipartite in structure. DNA encapsidation is compromised by changes in spacing between the two conserved parts of the consensus motif, rather than between different A repeats. Genetic evidence implicating packaging elements as independent units in viral DNA packaging is derived from the selection of revertants from a packaging-deficient adenovirus: multimerization of packaging repeats is sufficient for the evolution of packaging-competent viruses. Finally, we identify minimally sized segments of the adenovirus packaging domain that can confer viability and packaging activity to viruses carrying gross truncations within their left-end sequences. Coinfection experiments using the revertant as well as the minimal-packaging-domain mutant viruses strengthen existing arguments for the involvement of limiting, *trans*-acting components in viral DNA packaging.

Increasing the usefulness of adenovirus as a vector for the delivery of foreign genes into mammalian cells calls for the development of an in vitro packaging system to assemble recombinant viruses carrying a packaging signal fused to heterologous sequences. Such a packaging system could entail a number of advantages. First, the size of the foreign gene, which is restricted to about 10 kbp in order to propagate the virus in tissue culture (3), could be expanded substantially. Second, with all viral genes absent, the overwhelming immune response of the host to adenovirus infection potentially could be minimized. This response, both humoral and cytotoxic T-cell mediated, has mainly been ascribed to various viral gene products produced even in the context of replication-deficient second-generation adenovirus vectors (12, 32, 33). Little information is presently available about the basic mechanism of encapsidation of adenovirus DNA.

The assembly of adenovirus particles has been studied extensively by using a large number of viral temperature-sensitive mutants blocked at different stages of assembly at the restrictive temperature and by pulse-chase kinetic analyses (6–8, 10, 11). These early studies clearly established that adenovirus DNA is inserted into preformed, empty capsids late in the viral life cycle. Whether viral genomes enter the prohead in association with core proteins or as a separate entity is unclear, as is the exact structure of the viral DNA before and during entry. *cis*-acting packaging sequences in the adenovirus genome are required to direct selective encapsidation from the left end of the viral DNA. Polarity of adenovirus DNA packaging was

initially demonstrated in studies on viral incomplete particles containing viral DNA molecules of subgenomic length (5, 31), where a striking overrepresentation of left-end sequences was revealed, suggesting that DNA packaging occurs in a polar fashion from left to right. It was subsequently shown for adenovirus type 16 (Ad16) and Ad3 that a *cis*-acting packaging domain is located within the left 390 bp (19, 29). Sequence alignments of Ad3 with Ad5 and Ad12, representatives of adenovirus subgroups A, B, and C, revealed a large degree of sequence conservation within the interval between nucleotides (nt) 237 and 491 (25). This conservation is likely due to a conserved packaging signal in addition to a number of transcription factor binding sites located within this region. Conservation of the packaging sequences may reflect the dependence of all adenoviruses on a *cis*-acting encapsidation signal, suggestive of a similar mechanism of selective and polar DNA packaging for all adenovirus subgroups.

The *cis*-acting packaging domain in Ad5 is located in the left end 380 bp (16, 17, 20) and overlaps two distinct enhancer elements (Fig. 1A). Enhancer element I consists of a repeated sequence motif (21) and stimulates E1A transcription specifically upon binding of a cellular nuclear factor, EF-1A (4). Mutations in element I affecting its function can be efficiently complemented by propagation of the virus in 293 cells, a cell line that constitutively expresses the viral E1A and E1B gene products (18). Element II enhances transcription in *cis* from all early transcription units by an unknown mechanism (22). Element II mutations result in a decrease in early transcription and, since some of the early gene products are required for DNA replication, in a corresponding reduction in virus growth. This *cis*-acting defect can be efficiently complemented in *trans* by providing all of the early gene products in a mixed infection with wild-type virus (22). Deletion of the Ad5 packaging do-

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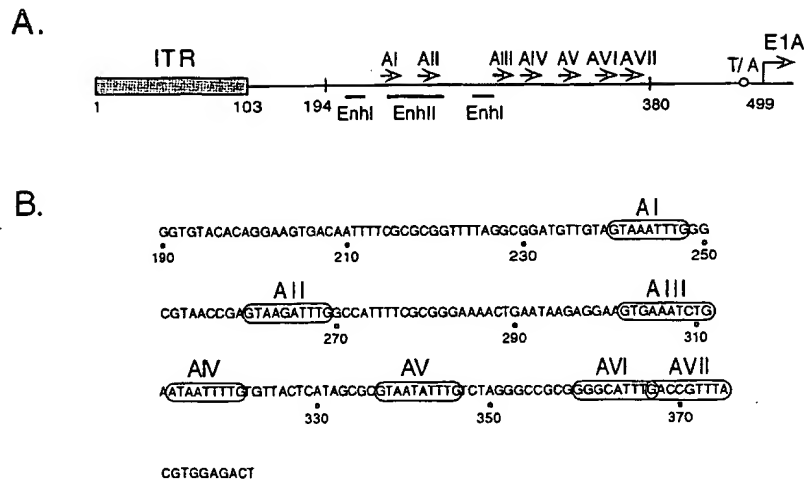
A.

B.

FIG. 1. (A) The Ad5 genomic left end and packaging domain. The schematic diagram shows the ITR, the packaging/enhancer region (nt 194 to 380), and the E1A 5' flanking region. Numbers indicate nucleotide positions relative to the left-end terminus. The packaging repeats (AI through AVII) are represented by arrows. The ITR is represented by a shaded box, and the E1A transcriptional start site is indicated by an arrow at nt 499. Transcriptional elements include a TATA box motif (T/A; black circle) and enhancer elements I and II (EnhI and EnhII; indicated by lines). (B) Nucleotide sequence of the Ad5 packaging domain. Numbers correspond to nucleotides relative to the left-end terminus. AI through AVII are circled.

main resulted in nonviability, but wild-type growth could be restored by the substitution of the left-end 353 bp at the right end of the genome (21). The Ad5 packaging domain was shown to be flexible with respect to its position as well as orientation within certain boundaries. However, it must be located within 600 bp of the inverted terminal repeat (ITR) (20). Whether ITR sequences themselves are required for adenovirus DNA packaging has not been determined yet due to a *cis* requirement for DNA replication.

Detailed analysis of the Ad5 packaging domain by deletion as well as linker scanning mutagenesis (16, 17) revealed that it consists of at least seven functional units called A repeats due to their AT-rich character (Fig. 1B). These elements are functionally redundant, but in spite of their redundancy, they follow a hierarchy of importance, with elements I, II, V, and VI as the functionally most dominant repeats. In support of a model implicating the A repeats as binding sites for limiting, *trans*-acting packaging components, cotransfection of an excess of packaging-domain sequences and wild-type adenovirus genomes resulted in a dramatic decrease in virus yield while DNA replication as well as late transcription were unaffected (17). This result is thought to reflect a competition event ongoing between unlinked packaging sequences and the viral genomes for the recognition of limiting packaging components. Consistent with this model is the fact that there are spatial constraints between individual packaging elements (17), presumably because the protein components involved have to interact with the packaging elements in a coordinate fashion. Notably, elements I and II as well as elements V and VI are separated by a spacing of 21 bp, or two helical turns of the DNA. Factors bound to these repeats would come to locate to the same side of the DNA double helix to interact with each other and/or components outside the packaging domain to mediate recognition of the prohead followed by DNA encapsidation.

Here we focus on an analysis of the 21-bp region separating A repeats I and II (AI and AII) and A repeats V and VI (AV and AVI). Mutagenesis within this region identifies additional sequence determinants as part of the packaging consensus motif. Of special importance is the presence of a CG dinucle-

otide located downstream of each of the four A repeats. Spacing between the consensus 5'-TTTG-3' and the CG located downstream, rather than spacing between individual A repeats, appears critical. We propose an extended consensus motif for the packaging elements which could represent a binding site for one or several packaging factors. In addition, we define minimal packaging sequences that exhibit maximal packaging activity in functional assays when introduced into a packaging domain-minus background. These minimal packaging domains may prove useful for the construction of new adenoviruses in the field of gene therapy. Finally, we selected revertants from a packaging-deficient adenovirus and show that in each case, A repeats were amplified, providing genetic evidence that these elements act as independent units in viral DNA packaging. The revertants exhibit a phenotype in which viruses containing more A repeats have a competitive advantage over viruses with fewer A repeats in coinfections with either wild-type virus or each other. These results again support an argument for the existence of limiting protein components as part of the packaging machinery.

MATERIALS AND METHODS

Virus was amplified and titers were determined on 293 cells. Mutant viruses were screened by restriction analysis of viral DNA obtained from infected 293 cells by the Hirt procedure (23). The authenticity of all linker scanning mutations was confirmed by amplification of viral left-end sequences spanning nt 1 to 1347 by PCR; the left-end fragments were gel purified and used as templates for the Double Stranded DNA Cycle Sequencing System (GIBCO BRL).

Cell lines and infections. 293 cells (18) and A549 cells, a human lung epithelium cell line, were maintained as monolayers in Dulbecco modified Eagle medium containing 10% bovine calf serum (HyClone). Virus stocks were generated by three freeze-thaw cycles of infected cell lysates, and titers were determined by plaque assays on 293 cells. Alternatively, virus particles were purified by CsCl equilibrium gradient sedimentation and titers were determined by optical density, whereby 1 optical density unit at A_{260} equals 10^{12} particles. Virus infections were performed at a multiplicity of infection of 3 PFU per cell or 200 particles per cell for 1 h at 37°C. Cells were then washed twice with Tris-buffered saline solution and overlaid with fresh medium.

Selection of revertants of a packaging-deficient adenovirus. The parent virus for the selection of revertants was constructed as follows. Plasmid pBR-194/455+AVI⁶ENH⁻ contains the Ad5 left-end Xba fragment carrying a deletion between nt 194 and 455, with six copies of an oligonucleotide containing AVI (see above) introduced at the deletion junction. Insertion of the AVI head-to-tail multimer created an Xho site at the right junction, which was used for the insertion of four copies of the E1A core enhancer element binding sites to ensure efficient E1A expression (2, 4). An EcoRI site separates the hexamer of AVI from the E1A core tetramer in the resulting plasmid, pBR-194/455+AVI⁶ENH⁺. The construct was then rebuilt into a dl309 virus background. The resulting virus had acquired two additional A repeats during the first growth cycle (see Results). To avoid recombination events between adenovirus left-end sequences integrated in the genome of 293 cells and parental genomes, the virus was subsequently propagated on A549 cells after initial recovery of plaques from 293 cells. After 12 passages of a number of independent isolates, an accelerated onset of cytopathic effect was evident. Putative revertants were plaque purified and amplified, and the revertant phenotype of each of the viruses relative to the parent was confirmed in single-infection and coinfection experiments. Left-end sequences of parent and revertant viral DNAs were compared by restriction and nucleotide sequence analysis as described above.

Determination of virus yield and packaging efficiency. To determine virus yield in a single infection, infected cell lysates were prepared 48 h postinfection, and the amount of infectious virus was determined by plaque assays on 293 cells. Packaging efficiency of the mutant viruses was determined in a coinfection of 293 cells with both mutant and wild-type dl309 virus. Forty-eight hours postinfection, one half of the cells was used to isolate total nuclear DNA; the other half was used for the preparation of viral DNA from purified virions as previously described (17). Briefly, total nuclear DNA was isolated from infected cells following the addition of Nonidet P-40 to 0.6% and precipitation of the nuclei by centrifugation. Viral DNA was prepared after lysis of infected cells in 0.2% deoxycholate and 10% ethanol at pH 9. Following treatment with RNase A and DNase, virions were lysed in 0.5% sarcosyl, and viral DNA was digested with proteinase K, phenol-chloroform extracted, and precipitated with ethanol. Both DNA preparations were digested with XbaI (XbaI and EcoRI for the revertant viruses and their parent) to distinguish between mutant and wild-type DNAs and quantitated by Southern blot hybridization (27) using pE1A-WT (21), ³²P labeled by the random primer method (13), as a probe. The relative intensities of the bands in autoradiograms were determined by densitometric scanning, using blots that were exposed to X-ray film without an intensifying screen. Quantitation of the data was performed by using the public domain NIH Image program (written by Wayne Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part no. PB93-504868). The data presented for virus yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of three to five independent experiments.
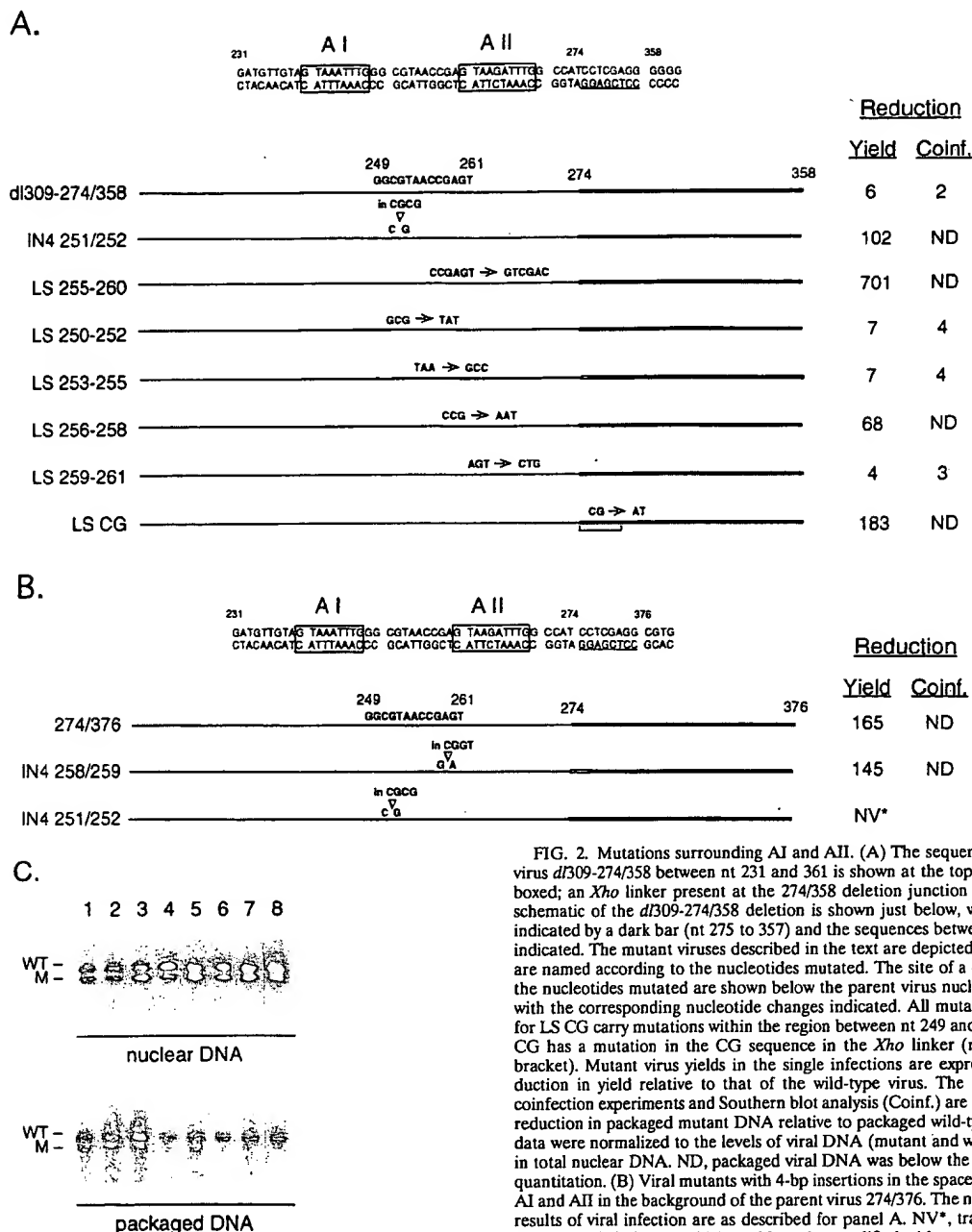
RESULTS

Spatial and sequence determinants important for viral DNA packaging in the spacer region between A repeats. As previously reported, changes of spacing between different A repeats, as well as between A repeats and regions outside the packaging domain, can have dramatic effects on viral packaging efficiency (16, 17). In this study, we focused on potential spacing requirements as well as sequence requirements within the two 21-bp spacer regions separating AI and AII, and AV and AVI. These four packaging elements have previously been implicated as the most relevant A repeats within the functional hierarchy of packaging elements (17). It was hypothesized that the separation of AI from AII and of AV from AVI by 21 bp, or two turns of the DNA double helix, may reflect a require-

ment for an interaction of trans-acting packaging components with packaging repeats on the same face of the DNA.

We constructed a panel of mutant viruses carrying linker scanning and insertion mutations within the 21-bp spacer regions between AI and AII as well as between AV and AVI. In view of the functional redundancy of the A repeats, we introduced all mutations between AI and AII in the context of a deletion of AIII, AIV, and AV (dl309-274/358). Mutations in the spacer region between AV and AVI were constructed in a mutant background with a deletion of AI, AII, and AIII (dl309-194/316). We tested all recombinant viruses in two independent assays. In the first assay, infectious virus yield was determined by plaque assays after a 48-h single infection of 293 cells. The adenovirus DNA packaging domain overlaps an enhancer element (element II) which stimulates transcription from all viral early transcription units in cis by an unknown mechanism. Mutations affecting the function of this element result in a decrease in viral DNA replication and consequently in a reduction of overall viral growth (22). This reduction (element II phenotype) can be complemented in a coinfection with wild-type virus providing all viral gene products in trans. To determine what portion of the reduction in overall growth as observed in the single infection is caused by a packaging defect, we based our second assay on a coinfection of 293 cells with the mutant and wild-type viruses. Total replicated DNA and packaged DNA were isolated from 293 cells after a 48-h infection. Mutant and wild-type DNAs were distinguished by restriction digestion, and their relative amounts were quantitated by Southern blot analysis. That way, the amount of packaged mutant virus DNA relative to the coinfecting wild-type DNA could be normalized to the levels of replicated DNA of each mutant and wild-type virus. This quantitation of the data enabled us to determine the reduction of viral DNA packaging independent of an element II phenotype whereby the coinfecting wild-type virus served as an internal control.

Figure 2A shows the mutations introduced into the spacer between AI and AII and summarizes the results obtained with these mutants. Figure 2C shows a Southern blot of a representative coinfection experiment. The parent virus, dl309-274/358, displayed a sixfold reduction in a single infection and a twofold decrease in packaging efficiency in a coinfection. A 4-bp insertion between AI and AII (IN4 251/252) resulted in a dramatic decrease in virus growth in a single infection, and packaged mutant viral DNA was not detectable in the coinfection. A 3-bp substitution overlapping the site of this insertion (LS 250–252) had no effect on virus growth compared to the parent virus. Mutant LS 255–260, carrying a 6-bp linker scanning mutation within the spacer, was dramatically defective in the single-infection and coinfection experiments. This result could reflect the fact that the first two nucleotides (GT) of the AII consensus sequence (17) were mutated; alternatively, it could implicate previously unrecognized sequences as part of the cis-acting packaging consensus motif. To distinguish between the two possibilities, 3-bp substitutions were introduced across the region between nt 249 to 261. Mutants LS 250–252, LS 253–255, and LS 259–261 grew and were packaged at the levels of the parent virus, whereas LS 256–258 displayed a 68-fold reduction in growth and packaged DNA was not detectable in a coinfection. This finding suggested that the defect observed with the mutant virus LS 255–260 was due to mutation of the three nucleotides CCG. The dinucleotide CG within this motif is located 11 nt downstream of the central thymidine in the thymidine triplet of AI. A CG dinucleotide is conserved in the same position relative to AI, AII, AV, and AVI (Fig. 1B), indicating that it may function in conjunction with the previously defined consensus packaging motif. The mutant virus LS

A.

		Reduction	
		Yield	Coinf.
dl309-274/358		6	2
IN4 251/252		102	ND
LS 255-260		701	ND
LS 250-252		7	4
LS 253-255		7	4
LS 256-258		68	ND
LS 259-261		4	3
LS CG		183	ND

B.

	Reduction	
	Yield	Coinf.
274/376	165	ND
IN4 258/259	145	ND
IN4 251/252	NV*	

C.

1 2 3 4 5 6 7 8

nuclear DNA

packaged DNA

FIG. 2. Mutations surrounding AI and AII. (A) The sequence of the parent virus dl309-274/358 between nt 231 and 361 is shown at the top. AI and AII are boxed; an Xho linker present at the 274/358 deletion junction is underlined. A schematic of the dl309-274/358 deletion is shown just below, with the deletion indicated by a dark bar (nt 275 to 357) and the sequences between nt 249 to 261 indicated. The mutant viruses described in the text are depicted underneath and are named according to the nucleotides mutated. The site of a 4-bp insertion or the nucleotides mutated are shown below the parent virus nucleotide sequence, with the corresponding nucleotide changes indicated. All mutant viruses except for LS CG carry mutations within the region between nt 249 and 261. Mutant LS CG has a mutation in the CG sequence in the Xho linker (represented by a bracket). Mutant virus yields in the single infections are expressed as fold reduction in yield relative to that of the wild-type virus. The results from the coinfection experiments and Southern blot analysis (Coinf.) are expressed as fold reduction in packaged mutant DNA relative to packaged wild-type DNA. These data were normalized to the levels of viral DNA (mutant and wild type) present in total nuclear DNA. ND, packaged viral DNA was below the level of accurate quantitation. (B) Viral mutants with 4-bp insertions in the spacer region between AI and AII in the background of the parent virus 274/376. The nomenclature and results of viral infection are as described for panel A. NV*, transfection experiments yielded plaques which could not be amplified without reversion to wild-type virus. (C) Southern blot analysis of viral DNA represented either in total nuclear DNA or in virion particles isolated from 293 cells coinfected with wild-type virus and the mutant viruses depicted in panel A. Total nuclear DNA or virion DNA was digested with XbaI and subjected to Southern blot analysis using an Ad5 left-end fragment as a ^{32}P-labeled probe. The corresponding left-end fragments of mutant (M) and wild-type (WT) genomes are indicated on the left. The mutant viruses tested were dl309-274/358 (lanes 1), LS 250-252 (lanes 2), LS 253-255 (lanes 3), LS 256-258 (lanes 4), LS 259-261 (lanes 5), LS 255-260 (lanes 6), IN4 251/252 (lanes 7), and LS CG (lanes 8).

CG emphasizes the importance of the CG located downstream of AII. During the construction of the parent virus dl309-274/358, the CG dinucleotide downstream of AII had originally been deleted but was coincidentally replaced by an Xho linker (16). Transversion of the dinucleotide in the mutant LS CG resulted in a dramatically defective phenotype of the mutant virus in single-infection and coinfection experiments, suggesting that even in the context of nonviral sequences, the presence of the dinucleotide CG downstream of AII is critical for viral DNA packaging.

Insertion of four nucleotides between AI and AII in mutant

IN4 251/252 dramatically affected viral growth and packaging efficiency. This mutation, however, alters both spacing between AI and AII and the spacing between AI and the CG dinucleotide downstream of it. To distinguish between these two possibilities, we rebuilt IN4 251/252 as well as a 4-bp mutation to

the right of the CG dinucleotide, IN4 258/259, into a mutant virus background lacking AIII, AIV, AV, AVI, and AVII (dl309-274/376 [Fig. 2B]). This mutant virus background was selected to exclude the possibility that effects on viral DNA packaging are not due to changes of spacing between AI and AII but instead are due to changes of spacing between AI and the remaining repeats to the right of AII (AVI and AVII) present in the dl309-274/358 parent virus. The results of single and coinfections with these viruses are displayed in Fig. 2B. The parent virus dl309-274/376 is 165-fold reduced in the single infection, and packaged mutant virus DNA is not detectable in the coinfection due to the large truncation of the packaging domain. Mutant 274/376:IN4 258/259 displayed a very similar phenotype, whereas mutant 274/376:IN4 251/252 was nonviable. These data emphasize that rather than a 21-bp spacing between AI and AII, the spacing between AI and the CG dinucleotide located 11 bp to the right appear to be critical for viral DNA packaging.

We introduced a similar set of mutations into the spacer region between AV and AVI in the context of a mutant virus that carries a deletion of AI through AIII (dl309-194/316). The results are summarized in Fig. 3A, and a representative Southern blot of a coinfection experiment is shown in Fig. 3B. The parent virus was reduced threefold in overall growth in a single infection, and its packaging efficiency was sixfold less than that of the coinfecting wild-type virus. Insertion of 6 nt into the CG located 11 bp to the right of AV resulted in a nonviable virus (IN6 355/356). Two mutant viruses, LS 354–359 and LS 354–356, with 6- and 3-bp substitutions overlapping the CG dinucleotide, respectively, also were nonviable. Their defective phenotypes implicate the CG dinucleotide downstream of AV as an important determinant for adenovirus DNA packaging in accordance with the previous set of mutants. In contrast to the previous results, however; mutation of the CG dinucleotide downstream of AVI (mutant LS 376–378) did not significantly affect viral packaging efficiency; overall growth in the single infection was reduced 21-fold, but virus growth in a coinfection was not affected. Most of the remaining 3-bp substitution mutants displayed a phenotype that is reminiscent of the element II phenotype described above. That is, defects observed in a single infection could be fully or largely complemented in a coinfection with a wild-type virus. The two most dramatic examples of this phenotype were caused by the mutations in LS 348–350 and LS 357–359. These mutants exhibited a small-plaque phenotype, which could, at least partially, account for the incongruities between the data from the single-infection and coinfection experiments. In spite of the generally more complex phenotypes observed with this panel of mutants, the presence of the dinucleotide CG downstream of AV appears to be necessary for viral DNA packaging as shown above for AI and AII. We suggest an extended consensus packaging motif for AI, AII, and AV, which in addition to the previously defined AT-rich motif contains a dinucleotide CG in position 11 and 12 downstream of the central thymidine of the invariable thymidine triplet (Fig. 4). This dinucleotide is also conserved downstream of AVI, but our mutational studies do not confirm its significance with respect to viral DNA packaging.

Defining minimal adenovirus packaging domains with maximal function in vivo. The adenovirus packaging repeats are functionally redundant, and in addition to the seven known A repeats, the presence of other packaging elements located outside the previously defined packaging domain has been suggested (17). For these reasons, it has been difficult to define a packaging domain of minimal size which is necessary and sufficient for adenovirus DNA packaging in vivo. We began our search for such a minimal packaging domain by deleting re-
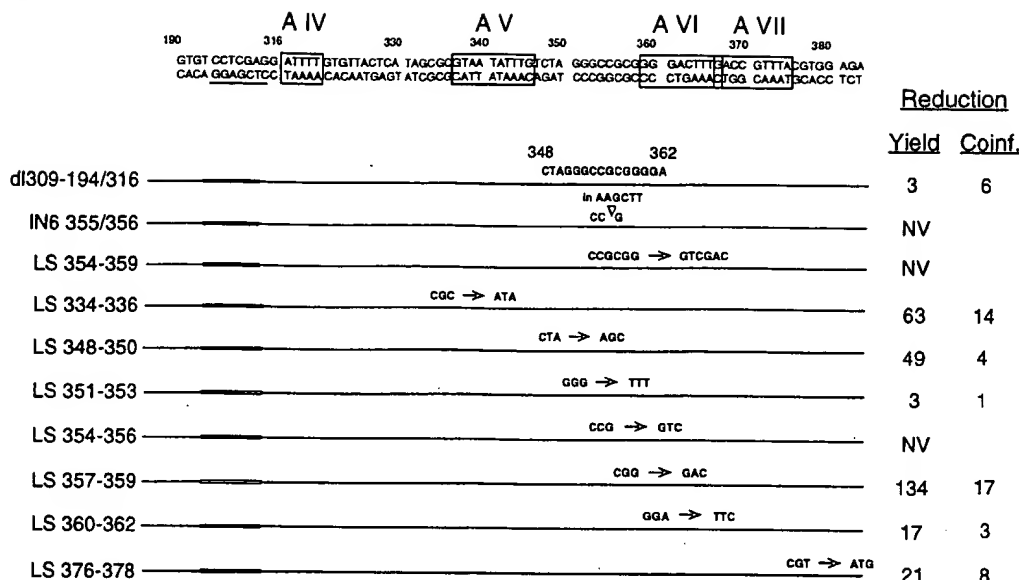
gions flanking the packaging domain in the context of a mutant virus 53/322, which carries only AV, AVI, and AVII. Figure 5 shows the parent virus 53/322 and two viruses derived from it, 53/322:640/814 and 53/322:455/814, which combine the 53/322 deletion with deletions extending from nt 640 to 814 and from nt 455 to 814, respectively. None of these viruses show significantly reduced abilities to package their DNA in a coinfection with wild-type virus. We conclude from these data that the regions between nt 53 to 322 and between nt 455 and 814 do not contain sequences with a critical function for viral DNA packaging in the context of a packaging domain that consists of AV, AVI, and AVII.

It has previously been demonstrated that the insertion of multimeric A repeats can rescue the nonviable phenotype of a virus lacking AI through AV (17). In a similar approach, we used a deletion of the entire packaging domain between nt 194 and 814 for the reinsertion of sequences that may function as minimal packaging domains (Fig. 5). The nt 814 position was chosen since previous results demonstrated that the Ad5 packaging sequences must be positioned within 600 bp of the left terminus (20). Thus functional packaging elements to the right of nt 800 appeared unlikely. Based on the results just described, we first introduced a monomer and dimer of a fragment containing AV, AVI, and AVII, which we will refer to as A(V-VII) and A(V-VII)^2, respectively. The fragments were inserted in both orientations. The parent virus 194/814 was nonviable, but viability could be restored by a monomeric and dimeric insert of AV, AVI, and AVII. The dimeric insert was significantly more effective at directing packaging than the monomeric insert, and packaging of the monomer was favored in the forward orientation relative to the reverse orientation. Importantly, the insertion of a forward dimer resulted in full restoration of packaging efficiency to wild-type levels. These data suggest that a dimer of AV, AVI, and AVII can function as a minimally sized, yet maximally functional, packaging domain in an orientation independent manner.

We next introduced (AV-VII)^2 into an even larger deletion background, eliminating sequences between nt 53 and 814. The nonviable phenotype of the parent virus 53/814 was also rescued by insertion of (AV-VII)^2 in either orientation. Packaging efficiency was three- to fourfold less than that of the coinfecting wild-type virus and was independent of the orientation of the insert. Overall growth in a single infection was reduced 82-fold in the forward, and 16-fold in the reverse, orientation. Again, the plaque size with these mutants was small, which may partly explain the large difference between single and coinfection results.

Selection of revertants from a packaging-deficient adenovirus. To generate a packaging-deficient virus as a parent for the selection of revertants, we constructed a virus with a deletion of sequences between nt 194 to 458 which eliminated the viral packaging domain (Fig. 1A). Six copies of AVI (21-bp oligonucleotide [see Materials and Methods]) were introduced at the site of the deletion, as well as four E1A enhancer repeats (2, 4) to ensure efficient E1A transcription (Fig. 6A). The parent virus was plaque purified from 293 cells, and a number of independent isolates were passaged on A549 cells to minimize the chance of recombination events between adenovirus left-end sequences integrated in the genome of 293 cells and parental genomes. During the first growth cycle on 293 cells, the parent virus had already acquired two additional copies of AVI (Fig. 6A); we therefore refer to it as 21/8 E1A$^+$. After 12 passages on A549 cells, an accelerated onset of cytopathic effect was evident. Viruses were plaque purified and amplified, and growth and packaging efficiencies of several independent isolates were determined on 293 cells. Figure 6A shows the

A.

A IV   A V   A VI   A VII

190   316   330   340   350   360   370   380

GTGT CCTCGAGG ATTTT GTGTTACTCA TAGCGC GTAA TATTTG CTA GGGCCGCG GG GACTTT ACC GTTTA CGTGG AGA
CACA GGAGCTCC TAAAA CACAATGAGT ATCGCG CATT ATAAAC AGAT CCCGGCGC CC CTGAAA TGG CAAAT GCACC TCT

Reduction

Yield Coinf.

                              348   362
dl309-194/316 ─────────────── CTAGGGCCGCGGGGA ────────────────── 3   6

                                   In AAGCTT
IN6 355/356 ───────────────────── CC▽G ──────────────────────── NV

                                   CCGCGG ─▶ GTCGAC
LS 354-359 ── NV

                              CGC ─▶ ATA
LS 334-336 ── 63   14

                                   CTA ─▶ AGC
LS 348-350 ── 49   4

                                   GGG ─▶ TTT
LS 351-353 ── 3   1

                                   CCG ─▶ GTC
LS 354-356 ── NV

                                   CGG ─▶ GAC
LS 357-359 ── 134   17

                                   GGA ─▶ TTC
LS 360-362 ── 17   3

                                        CGT ─▶ ATG
LS 376-378 ── 21   8

B.

1 2 3 4 5 6 7

WT –
M –

nuclear DNA

WT –
M –

packaged DNA

results of single infections and coinfections of the parent virus and four revertants. The parent virus 21/8 E1A[+] was reduced 32-fold in growth in a single infection relative to the wild type. Packaging efficiency was reduced correspondingly in a coinfection with wild-type virus. Left-end sequences of the revertants were analyzed by restriction digestion and sequencing and were found to harbor precise amplifications of AVI, with 12, 16, 17, and 20 elements present, compared to 8 elements in the parent virus. All revertant viruses exhibited a significant (11- to 16-fold) increase of viral growth in the single infection and a comparable increase of packaging efficiency in the coinfection relative to the parent virus, indicating that amplification of AVI provided the viruses with a competitive advantage. The fact that revertants of 21/8 E1A[+] evolved through the amplification of A repeats presents genetic evidence that these sequences function as independent units in viral DNA packaging.

An example of a Southern blot prepared from 293 cells coinfected with wild-type virus and the parent virus (21/8 E1A[+]) or its revertants is shown in Fig. 6B. The relative mobility of the left-end fragment varies between the parent virus
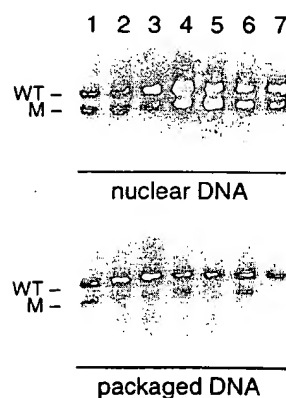
FIG. 3. Mutations surrounding AV and AVI. (A) Mutant viruses with substitution mutations and an insertion mutation in the region between AV and AVI, as well as substitution mutations to the left of AV and to the right of AVII. The nomenclature and the results from single infections and coinfections are as described for Fig. 2A. NV, mutant virus was nonviable in at least three transfection experiments. (B) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with wild-type (WT) virus and the mutant (M) viruses depicted in panel A (performed as described in the legend to Fig. 2C). The mutant viruses analyzed were LS 334–336 (lanes 1), LS 348–350 (lanes 2), LS 351–353 (lanes 3), LS 357–359 (lanes 4), LS 360–362 (lanes 5), and LS 376–378 (lanes 6).

and the revertants, depending on the number of copies of AVI present. Each of the revertants packaged viral DNA in the coinfection experiment with increased efficiency relative to the parent virus. The faint band above the AVI fragment with the parental virus (Fig. 6B, lane 1) presumably represents genomes with already multimerized packaging repeats, suggesting a strong evolutionary pressure for the presence of more than eight copies of AVI. We also tested the packaging abilities of the parent virus and of the revertant viruses carrying 12, 16, and 17 copies of AVI in a coinfection with the revertant car-

A I   A II
TTAGGCGGATGTTGT AGTAAATTTG GGCGTAACCGA GTAAGATTTG GCCATTTTCGCGGGAA
  I   I II   I   I IIIII   III I   IIII II I II II II I
TGTT ACTCATAGCGC GTAATATTTG TCTAGGGCCGC GGGGACTTTG ACCGTTTACGTGGAGA
A V   A VI

A I   AGTAA ATTTG GGCGTAAC CG AGTAAG
A II GTAAG ATTTG GCCATTTT CG CGGGAA
A V   GTAAT ATTTG TCTAGGGC CG CGGGGA
A VI GGGGA CTTTG ACCGTTTA CG TGGAGA
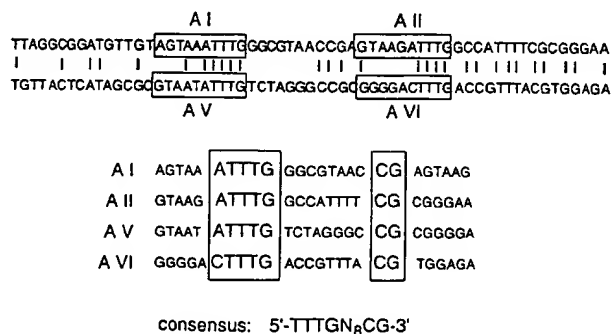
consensus: 5'-TTTGN8CG-3'

FIG. 4. Alignment of AI, AII, AV, and AVI. The packaging repeats are boxed; invariable nucleotides are indicated by lines between the sequences. Shown below is an alignment of the individual A repeats. Invariant nucleotides between these repeats are indicated by large letters. A new consensus motif for the Ad5 packaging elements is shown at the bottom.
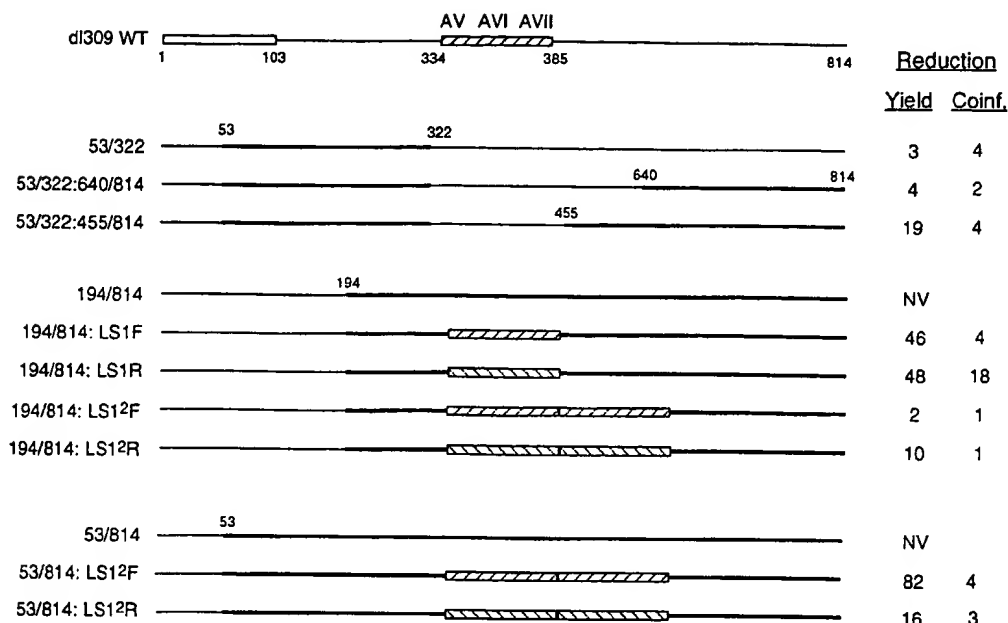
AV AVI AVII

dl309 WT

1   103   334   385   814

Reduction

Yield Coinf.

53/322   53   322   3   4

53/322:640/814   640   814   4   2

53/322:455/814   455   19   4

194/814   194   NV

194/814: LS1F   46   4

194/814: LS1R   48   18

194/814: LS12F   2   1

194/814: LS12R   10   1

53/814   53   NV

53/814: LS12F   82   4

53/814: LS12R   16   3

FIG. 5. Viruses with minimal packaging domains. A schematic representation of left-end sequences of dl309 wild-type (WT) virus is shown at the top. The ITR is represented by an open box; AV through AVII (nt 334 to 385) are represented by a hatched box. Numbers represent nucleotide positions relative to the left end. Deletions in individual viruses are indicated by a solid line; the last nucleotides remaining on either side of the deletion are indicated and are numbered relative to the left-end terminus. The names of the mutant viruses in the top set represent the deletions introduced into left-end sequences. The middle and bottom sets of mutant viruses contain deletions between nt 194 and 814 and nt 53 and 814, respectively. A monomer (AV-VII) or dimer (AV-VII)^2 of an oligonucleotide containing AV through AVII was inserted at the deletion site junction. The orientation of the AV-VII fragment is indicated as forward (F) or reverse (R). The results of single and coinfections of 293 cells are expressed as described in the legend to Fig. 2A.

rying 20 A repeats. These viruses differ from each other only by the copy number of AVI present in place of the viral packaging domain. Figure 6C shows Southern blots prepared from total nuclear DNA as well as packaged DNA from these coinfections. The uppermost band is shared by all viruses and is composed of the four E1A enhancer repeats in conjunction with the region between nt 458 and 1339. The lower fragments contain nt 1 to 194 as well as the AVI multimers. All viruses replicated to equal levels in a coinfection with the 21/20 E1A$^+$ virus (lanes 1 to 4). When packaged DNA was analyzed, however, packaging of the parent virus as well as the revertant virus with 12 A repeats was substantially reduced (lanes 5 and 6). Viruses harboring 16 and 17 copies of AVI packaged their DNA as efficiently as the 21/20 E1A$^+$ revertant (lanes 7 and 8). These data strongly support the notion that viruses with more packaging elements have a competitive advantage over viruses with fewer elements when they are challenged to compete with each other. All four revertants, when tested in a single infection, exhibited similar growth properties (Fig. 6A), which strengthens our hypothesis that trans-acting packaging components are required to bind the packaging repeats before DNA encapsidation can ensue. Such packaging factors may be limiting, which would result in preferential encapsidation of genomes containing more packaging elements in a coinfection experiment.

We also inserted 12 head-to-tail copies of AVI in either orientation into the 53/814 deletion as shown in Fig. 7. The resulting mutant viruses, 194/814:AVI/12F and 194/814:AVI/ 12R, were 22- and 48-fold reduced in the single infection, with a corresponding decrease in the coinfection of 17- and 21-fold. Sequencing of viral left-end fragments revealed more than 12 copies of AVI in both viruses, indicating that an amplification

of packaging elements had taken place. At least 18 copies were present in mutant 194/814:AVI/12F, and at least 15 copies were present in mutant 194/814:AVI/12R. This amplification event is reminiscent of the multimerization of A repeats in revertants selected from a packaging-deficient mutant virus described above. These data confirm the results obtained with the selection of revertants carrying amplified packaging repeats.

DISCUSSION

Adenovirus DNA packaging is often likened to DNA packaging in double-stranded DNA bacteriophage systems such as phage lambda or φ29. Viral DNA is specifically selected from the pool of total DNA for its packaging, and insertion of the genome into preformed, empty capsids proceeds in a polar fashion. For these two phages, it has clearly been shown that phage-encoded packaging factors selectively bind packaging sequences located within the genomic left-end terminus. Formation of a nucleoprotein complex on the packaging domain then marks the respective molecule as a bone fide packaging substrate followed by recognition of the empty capsid and insertion of the DNA with left-to-right polarity (1, 9).

The identities of such packaging factors in the case of adenovirus are still unknown, but several lines of evidence strongly support the existence of limiting trans-acting packaging components. A number of packaging mutants were previously reported to exhibit a greater reduction in packaging efficiency in the coinfection experiment than was expected from single infections with the respective viruses. A model was proposed in which a competition for a limiting trans-acting packaging component would take place between mutant and wild-type vi-

A.

B.

C.

FIG. 6. Adenovirus packaging revertants. (A) Schematic representation of a packaging-deficient parent virus and the revertant viruses selected from it. The E1A transcriptional start site at nt 499 is represented by an arrow; E1A enhancer sequences are indicated by closed circles; open boxes represent a 21-bp oligonucleotide containing AVI (see Materials and Methods). The parent plasmid carried six AVI copies (21/6 E1A⁺). The parental virus originally isolated had acquired two additional copies of AVI during the first round of growth (21/8 E1A⁺). AVI was further amplified upon multiple passages of the 21/8 E1A⁺ parent virus, as indicated by the names of the revertant viruses and the number of open boxes in the diagram. The data from single infections as well as coinfections of 293 cells are expressed as described in the legend to Fig. 2A. (B) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with wild-type virus and parent or revertant (rev.) viruses. Viral genomes were digested with XbaI and EcoRI and subjected to Southern blot analysis as described the legend to Fig. 2C. The wild-type (WT) Xba fragment is indicated on the left. The parent and revertant viruses carry an EcoRI site between the AVI multimers and the E1A enhancer repeats. Correspondingly, whereas the upper fragment is of equal length for all of these viruses, fragments representing the left-end terminus vary in mobility depending on the copy number of AVI present. These left-end fragments containing multiple copies of AVI are indicated on the left. The mutant viruses tested were the parent virus 21/8 (lanes 1) and the revertant viruses 21/12 E1A⁺ (lanes 2), 21/16 E1A⁺ (lanes 3), 21/17 E1A⁺ (lanes 4), and 21/20 E1A⁺ (lanes 5). (C) Southern blot analysis of total nuclear and virion DNA isolated from 293 cells coinfected with the revertant carrying 20 copies of AVI and its parent virus carrying eight A repeats or other revertants carrying 12, 16, and 17 copies. Southern blot analysis was performed as described in the legend to Fig. 6B. Fragments representing the left-end termini of the different viruses are indicated on the left.

ruses, with the wild-type packaging domain providing a competitive advantage (16). This model was strongly supported by a cotransfection experiment in which an excess of unlinked packaging-domain sequences dramatically inhibited viral growth without affecting DNA replication and late transcription, presumably by titrating packaging factors away from virus genomes (17). A third line of evidence is presented in this report and involves the evolution of packaging-competent viruses from a packaging-deficient parent virus through the amplification of packaging repeats (Fig. 6A). Twelve, 16, 17, and 20 copies of A repeats in place of the packaging domain resulted in an improvement of virus growth and packaging ability to wild-type or near-wild-type levels. When challenged in a coinfection experiment, viruses carrying more A repeats displayed a competitive advantage over viruses with fewer repeats (Fig. 6C). Specifically, 12 copies of AVI did not allow significant levels of packaging in a coinfection with a virus containing 20 A repeats, whereas 16 and 17 copies did. It appears likely that this is the result of a competition for the binding of a limiting packaging component in which more packaging elements increase the likelihood of binding of such a component.

Our results provide genetic evidence that packaging elements, in this case AVI, act as independent functional units in viral DNA packaging. To ensure that the reverted phenotype is a direct consequence of the presence of additional copies of A repeats and not due to unidentified second-site mutations, we reconstructed a recombinant adenovirus carrying 12 copies of AVI inserted into a deletion between nt 194 and 458. Left-end sequences of this recombinant virus including the packaging domain are identical to the 21/12 E1A⁺ revertant left end. As predicted, in a single infection this recombinant virus grew to the same level as the revertants carrying 12 or more A repeats (data not shown). This result argues against the possibility that revertants carried other second-site mutations that augmented packaging efficiency. An alternative explanation for the stimulation of DNA packaging through the amplification of packaging repeats is an attempt to optimize the spacing between the packaging elements and the left-end terminus of the adenovirus genome. This possibility appears unlikely for two reasons. First, a DNA fragment containing AV, AVI, and AVII functioned efficiently for packaging as a dimer when positioned at nt 53 (Fig. 5). Amplification of this segment was not observed, and so packaging elements can function when located near the terminus of the viral genome. Second, amplification of A repeats was observed when multimers of AVI were located at nt 53 or 194 (Fig. 6 and 7). The fact that selective pressure for the multimerization of A repeats exists in two situations in which spacing between the packaging domain and the left-end terminus is substantially different argues against a selection to make specific spatial changes and for a selection for the pres-

FIG. 7. AVI specifies packaging. A schematic representation of left-end sequences of dl309 wild-type virus is shown at the top. The ITR is represented by an open box; AVI (nt 354 to 370) is represented by a hatched box. Numbers represent nucleotide positions relative to the left end. The mutant viruses contain a deletion between nt 53 and 814 and the insertion of 12 copies of AVI with mutants 194/814:AVI/12 in the forward (F) or reverse (R) orientation. The results of single infections and coinfections of 293 cells are expressed as described in the legend to Fig. 2A.

ence of more packaging elements. The two possibilities, however, are not mutually exclusive.

In addition to a packaging factor(s) presumed to bind the A repeats, factors bound to ITR sequences also have been hypothesized to play a role in viral DNA packaging. The adenovirus packaging domain, although positionally flexible to a certain extent, has to be located near the ITR to maintain activity (20). Two mutant viruses define the minimal ITR sequences that may be involved in DNA packaging [Fig. 5, 53/814:(AV-VII)²F and 53/814:(AV-VII)²R]. These viruses carry packaging domains in the context of the 53/814 deletion and display near-wild-type packaging efficiency. Only the left-most 53 nt, the core replication sequences, are present to the left of the packaging domain with these mutant viruses. Therefore, if factors bound to the left-end terminus do function in viral DNA packaging, they would have to serve a dual function in DNA replication as well as encapsidation of the genome. In support of the possible involvement of replication factors in adenovirus DNA packaging, certain mutations in the adenovirus terminal protein (TP), which is covalently attached to the 5′ terminus of the viral genome, inhibit virus growth in vivo but do not diminish terminal protein replication function in vitro (14, 15, 28). Such a result is consistent with a dual role for TP in replication and viral DNA packaging. Interestingly, in the case of bacteriophage φ29, which especially resembles adenovirus by way of a protein-primed DNA replication mechanism and the polar encapsidation of a nonconcatemeric genome, the TP primer for DNA replication, gp3, is also an enhancer of DNA packaging (1).

We attempted to define minimal packaging sequences with maximal packaging activity in vivo. A dimer of a fragment containing AV, AVI, and AVII rescued packaging efficiency in a coinfection experiment to wild-type levels in the 194/811 deletion mutant background and to near-wild-type levels in the 53/811 mutant background (Fig. 5). Therefore, this fragment constitutes the minimal packaging domain so far defined which is necessary and sufficient for viral DNA packaging in vivo. The AV-VII dimer rescued viral packaging significantly better than a multimer of AVI in the 53/811 mutant background (compare Fig. 5 and 7). Previous mutational studies identified AVI as the functionally most important packaging element in the context of a virus lacking AI through AIII (17). The fact that multimers of AVI could not reconstitute viral packaging to the extent that a combination of AV, AVI, and AVII did, even with a higher copy number of repeats present, suggests that a combination of different A repeats supports viral packaging better than only one type of element. This in turn could implicate more than one protein component to be part of the viral packaging machinery. To achieve maximal packaging efficiency, distinct factors would be required to bind different packaging elements followed by recognition of the prohead and subsequent encapsidation. The definition of minimal packaging domains with maximal packaging activity in vivo may prove useful for the construction of new helper viruses in the field of gene therapy to minimize target sequences for homologous recombination between gene therapy and helper virus as well as to maximize insert size.

It was previously established that adenovirus packaging repeats are functionally redundant (16, 17). Despite this redundancy, individual elements are not functionally equivalent but follow a hierarchy of importance. Early alignments identified a loosely conserved consensus motif, $GTN_{3-4}TTTG$, for the packaging elements. These findings were based on extensive analyses of viruses carrying deletion mutations as well as linker scanning mutations overlapping individual packaging elements in the context of a minimal packaging domain (16, 17). AI and
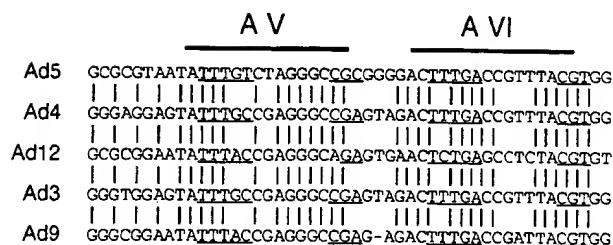

FIG. 8. Alignment of putative packaging repeats in different adenovirus subgroups. The nucleotide sequences corresponding to AV and AVI are shown for Ad5 (subgroup C), Ad4 (subgroup E), Ad12 (subgroup A), Ad3 (subgroup B), and Ad9 (subgroup D). The positions of AV and AVI in Ad5 are shown by lines above the sequences. Nucleotides identical between all subgroups are indicated by vertical lines.

AII, as well as AV and AVI, are the functionally dominant repeats, and they are also separated from each other by 21 bp. Factors bound to these repeats would be located on the same side of the DNA double helix to potentially interact with each other and/or with factors bound to the leftmost 53 nt of the adenovirus genome to allow for DNA encapsidation. In studies to address the importance of 21-bp spacing between AI and AII, as well as AV and AVI (Fig. 2 and 3), we identified a CG dinucleotide, located in the identical position relative to AI, AII, AV, and AVI, that is 11 bp downstream of the middle thymidine residue within the thymidine triplet (Fig. 4). Our mutational studies suggest that the 11-bp spacing between the TTTG and the downstream CG is important but that the 21-bp spacing between different packaging elements is not required, at least not with respect to AI and AII (Fig. 2B). An alignment of the four functionally dominant packaging repeats together with our mutational studies defines a more extended consensus motif, $5′-TTTGN_8CG-3′$ (Fig. 4). Interestingly, these sequences also are conserved between different adenovirus subgroups in the regions corresponding to AV and AVI (Fig. 8). In vivo, the presence of the CG dinucleotide was found to be critical downstream of AI, AII, and AV but not downstream of AVI (Fig. 2 and 3). It is possible that functional redundancy applies not only to the AT-rich part of the new consensus motif but also to the CG sequence.

A single trans-acting packaging factor could interact with both conserved parts of the consensus motif, and it is noteworthy that the 11-bp spacing between the TTTG and CG sequences would present both halves of the consensus sequence on the same face of the DNA helix. Alternatively, the bipartite nature of the consensus motif could imply that distinct factors bind to the two conserved regions. Since different individual packaging repeats or combinations of repeats do not appear to be functionally equivalent, different packaging factors may interact with different individual A repeats. If one or more host cell factors are involved in adenovirus packaging, this may reflect the ability to recruit different binding proteins depending on the cell type infected and the predominance of the respective cellular factors in that cell type. Consequently, functional redundancy of the packaging elements could constitute one of many strategies that the virus has developed to allow for its well-documented ability to infect a wide variety of cell types.

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